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U-21,963, a New Antibiotic

I. Discovery and Biological Activity

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ABSTRACT

PYKE, THOMAS R. (The Upjohn Co., Kalamazoo, Mich.), AND ALMA DIETZ. U-21,963, a new antibiotic. I. Discovery and biological activity. Appl. Microbiol. **14**:506–510. 1966.—A new antibiotic, U-21,963, is produced by a new strain of *Trichoderma viride*. Antibiotic activity can be demonstrated against both gram-positive and gram-negative bacteria and also against a wide variety of fungi. U-21,963 is not cross-resistant with other commonly used antibiotics. U-21,963 afforded no protection against *Klebsiella pneumoniae*, *Streptococcus pyogenes*, or *Staphylococcus aureus* when it was injected subcutaneously into mice.

Trichoderma viride produces a new antibiotic designated U-21,963. This paper describes the organism which produces U-21,963, and some of the biological properties of the antibiotic. An accompanying paper describes the isolation and characterization of U-21,963 (4).

MATERIALS AND METHODS

U-21,963 is produced by a strain of *T. viride*, designated UC 4785, isolated from soil. The organism was identified by microscopic appearance, cultural characteristics, and comparison with known cultures of *T. viride*. Seed flasks were inoculated with spore preparations of the culture which were maintained in soil or from vegetative mycelium preserved under liquid nitrogen. The culture was incubated at 28 C for 48 hr in a seed medium consisting of glucose monohydrate (Cerelose), 25 g per liter; and Pharmamedia, 25 g per liter. The vegetative seed was used at the rate of 5% to inoculate a fermentation medium consisting of liquid peptone (Wilson Laboratories, Chicago, Ill.), Malt Extract (Difco), and glucose monohydrate (presterilization *p*H, 6.8). A 0.5-ml amount of lard oil was added to each 100 ml of medium to control foam production. Shaken-flask fermentations were run in 500-ml Erlenmeyer baffled flasks containing 100 ml of medium, and were incubated at 25 C on a Gump rotary shaker operating at 250 rev/min with a 2.5-inch (6.4-cm) stroke.

U-21,963 was differentiated from other antibiotics by its antibacterial and antifungal spectra and by paper chromatography. The antibiotic was spotted on Whatman no. 1 filter paper and developed without prior equilibration by use of the descending method. The activity was located by plating the developed strips on trays of agar seeded with *Sarcina lutea* (ATCC 9341).

U-21,963 levels were assayed with a standard disc-plate agar diffusion assay. Samples (0.08 ml) in 0.1 M phosphate buffer (*p*H 5.0) were placed on 12.7-mm paper discs and were assayed against *S. lutea*. The results were given in biounits—a biounit being defined as the amount of antibiotic necessary to give a 20-mm zone of inhibition after 20 hr of incubation. The in vitro spectrum of U-21,963 was determined by two-fold dilution end points in Brain Heart Infusion (Difco) broth. End points were read after 20 hr of incubation at 32 C.

The in vivo activity of U-21,963 was determined by the method of Lewis et al. (3). White male mice (CF-1) were infected with a 100 LD_{50} dose of the organism to be tested. The infected animals were treated with the compound either subcutaneously (0.2-ml volume) or orally (0.5-ml volume) on 4 successive days. At the end of 7 days, the efficacy of U-21,963 was judged by relating the mortality-survivor ratio of the treated animals to the mortality-survivor ratio of an untreated group.

RESULTS AND DISCUSSION

Taxonomy. Webster and Lomas (6) discussed some of the taxonomic differences between the genera *Trichoderma* and *Gliocladium*. The type of conidiophore branching found in the producing culture appears to be more closely related to the open form seen in the genus *Trichoderma* than to the appressed type occurring in *Gliocladium*. The organism is both culturally and morphologically within the limits outlined for the genus *Trichoderma* by Bisby (1), Cooke (2), and Smith and Raistrick (5), and was therefore identified as *T. viride* Pers. ex Fries. The growth characteristics of the U-21,963-producing culture on five media in comparison with three authentic isolates of *T. viride* are shown in Table 1. Figure 1 shows a comparison of UC 4785 and three authentic *Trichoderma* isolates on six

TABLE 1. *Macroscopic appearance of UC 4785 and three authentic Trichoderma viride cultures*

Agar medium	UC 4785	Illinois 96, UC 1400	NRRL 1762, UC 4021	BB-113, UC 4328
Neopeptone-dextrose	Cottony white aerial growth Pale yellow reverse	Cottony white aerial growth turning green Pink-tan reverse	Heavy cottony white aerial growth turning green Tan reverse	Sparse cottony white aerial growth with compacted green growth on periphery Pale yellow reverse
Czapek Solution	Very cottony white aerial growth Pale cream reverse	Cottony white aerial growth becoming green cottony to compact Gray-green reverse	Cottony white aerial growth becoming compacted green Gray-green reverse	Compacted green aerial growth Gray-green reverse
Leonian	Cottony white aerial growth becoming compacted green Gray-green reverse	Cottony white aerial growth becoming cottony to compacted green Gray-green reverse	Cottony white aerial growth becoming compacted green Gray-green reverse	Cottony white aerial growth becoming compacted green Gray-green reverse
Water	Spotty white aerial growth turning to spotty compact green Trace gray-green reverse	Sparse cottony white aerial growth Colorless reverse	Spotty white aerial growth turning to spotty compact green Trace gray-green reverse	Spotty white aerial growth turning to spotty compact green Trace gray-green reverse
Potato Dextrose	Cottony white aerial growth becoming compacted green Gray-green reverse	Cottony white aerial growth becoming smooth green Gray-green reverse	Cottony white aerial growth becoming compacted green Gray-green reverse	Cottony white aerial growth becoming compacted green Gray-green reverse

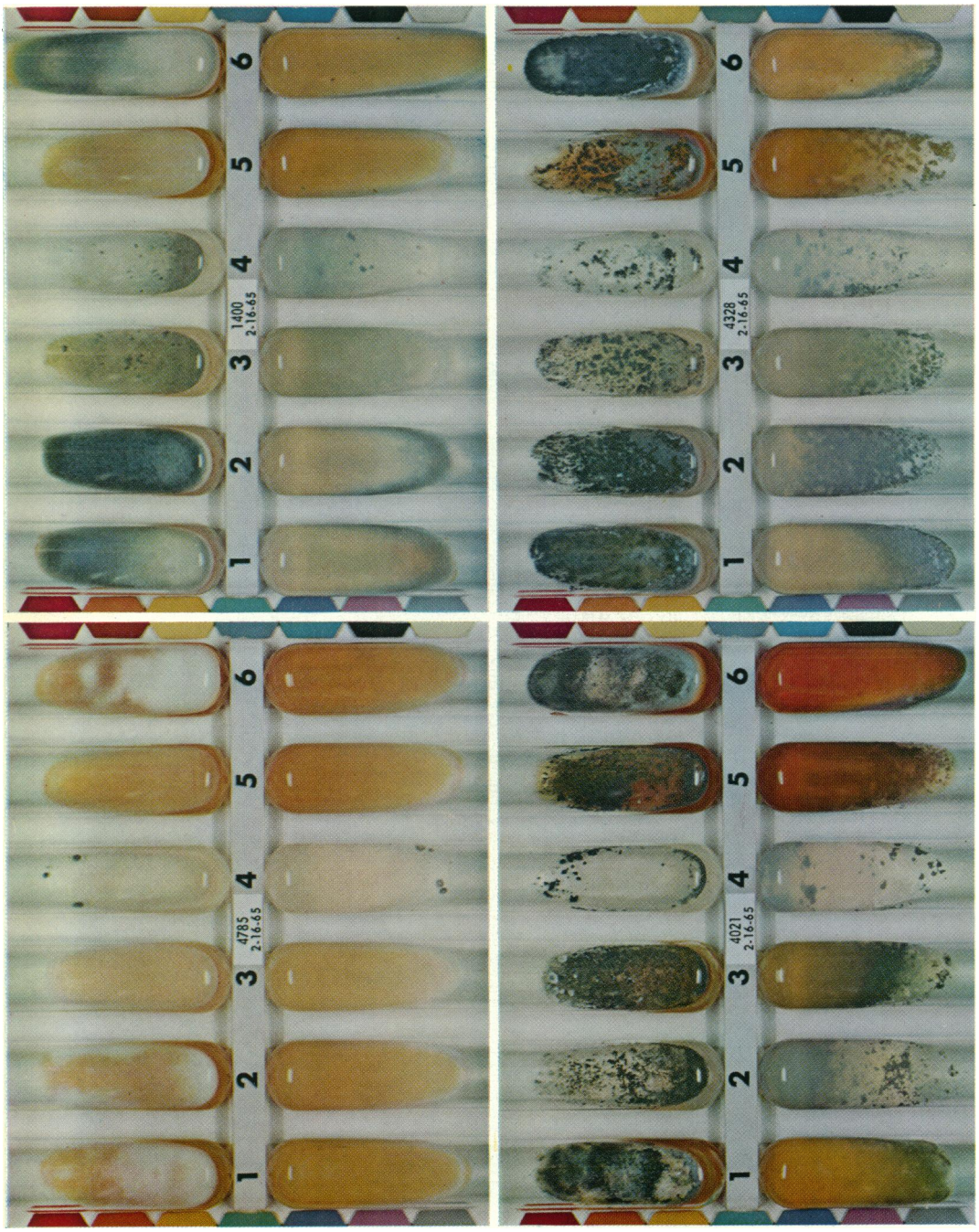

FIG. 1. *Comparison of UC 4785 with three authentic strains of Trichoderma viride. The numbers 4785, 1400, 4021, and 4328 given on the figure are UC numbers. UC 1400, UC 4021, and UC 4328 are strains Illinois 96, NRRL 1762, and BB-113, respectively. Incubation was for 7 days on the following agar media:* (*1*) *Neopeptone-dextrose;* (*2*) *Czapek sucrose (Difco);* (*3*) *Leonian;* (*4*) *water;* (*5*) *Potato Dextrose (Difco);* (*6*) *Gray.*

TABLE 2. *Fermentation titer and pH pattern of U-21,963-producing strain of Trichoderma viride*

Age	U-21,963 titer (biounits/ml)	pH
hr		
0	0	6.3
48	24	4.6
72	21	4.6
96	10	4.5
120	9	5.4

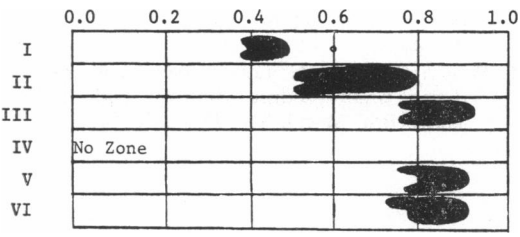

FIG. 2. *Bioautographic characteristics of U-21,963. The solvent systems used were as follows: (I) 1-butanol–water (84:16), 16 hr; (II) 1-butanol–water (84:16) plus 0.25% p-toluenesulfonic acid, 16 hr; (III) 1-butanol–acetic acid–water (2:1:1), 16 hr; (IV) 2% piperidine (v/v) in 1-butanol–water (84:16), 16 hr; (V) 1-butanol–water (4:96), 5 hr; (VI) 1-butanol–water (4:96) plus 0.25% p-toluenesulfonic acid, 5 hr.*

TABLE 3. *In vitro end points of U-21,963 in Brain Heart Infusion broth*

Organism	Minimal inhibitory concn*
	μg/ml
Staphylococcus aureus UC 552	125
S. aureus UC 76	250
S. aureus UC 70	125
Streptococcus pyogenes UC 152	250
S. faecalis UC 157	250
Klebsiella pneumoniae UC 58	125
Escherichia coli UC 51	125
Proteus vulgaris UC 93	500
Pseudomonas aeruginosa UC 95	>1,000
Salmonella paratyphi UC 263	125
S. gallinarum UC 265	62
S. typhosa UC 215	62

* Amount (micrograms) of free acid equivalent activity.

diagnostic agar media. (The *Trichoderma* isolates used for comparison were: Illinois 96 from the University of Illinois; NRRL 1762 from the Northern Utilization Research and Development Division, Agricultural Research Service, Peoria, Ill.; BB-113 from London School of Tropical Hygiene and Medicine, London, England.)

TABLE 4. *Antifungal activity of U-21,963 on Gray's agar medium incubated at 24 C for 72 hr**

Test fungus	Minimal inhibitory concn†
	μg/ml
Coccidioides immitis	1,000
Geotrichum sp.	1,000
Hormodendrum compactum	1,000
Phialophora verrucosa	1,000
Histoplasma capsulatum	1,000
Sporotrichum schenkii	1,000
Monosporium apiospermum	1,000
Candida albicans	1,000
Trichophyton mentagrophytes	1,000
Glomerella cingulata	1,000
Graphium fructicolum	1,000
Penicillium italicum	1,000
Aspergillus fumigatus	1,000
Alternaria solani	1,000
Verticillium sp.	1,000
Monolinia fructicola	1,000
Sclerotinia sclerotiorum	1,000
Rhizoctonia solani	1,000
Nocardia asteroides	100
Blastomyces dermatitidis	100
Cryptococcus neoformans	100
Trichophyton rubrum	100
Trichophyton violaceum	100
Penicillium oxalicum	10

* The antibiotic was incorporated into the medium at 1,000, 100, 10, and 1 μg/ml.

† Amount (micrograms) of free acid equivalent activity.

Paper chromatography. Figure 2 shows the paper chromatographic pattern of U-21,963 in six solvent systems. U-21,963 was distinguishable from all available similar antibiotics.

Fermentation studies. Table 2 shows a typical fermentation pattern. Fermentations at 28 and 32 C were less active than those at 25 C.

In vitro spectrum. Table 3 lists the in vitro bacterial spectrum of U-21,963 in Brain Heart Infusion broth (*p*H 7.4), and Table 4 shows the results of antifungal testing. The preparation was estimated to be 25% pure. No cross-resistance was found between U-21,963 and any of the commonly used antibiotics. Of eight *Staphylococcus aureus* clinical isolates tested, four were resistant to penicillin, but all were sensitive to U-21,963.

In vivo testing. Mice infected with *Klebsiella pneumoniae* received some protection with a subcutaneous dose of 320 mg of U-21,963 per kg, but mice infected with *Escherichia coli* were

not protected. Toxicity as evidenced by weight loss occurred at this level. More highly purified material was toxic at 40 mg/kg and failed to protect mice infected with *K. pneumoniae*. No blood levels could be demonstrated at 800 mg/kg orally or 320 mg/kg subcutaneously.

Other tests. U-21,963 was inhibitory to KB tumor cells in an in vitro test.

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